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14. ABSTRACT In metastatic prostate cancer there is increased frequency of loss of genetic material from chromosome 18q. We previously identified two regions on chromosome 18q as the potential sites of tumor suppressor genes involved in metastasis suppression. To identify these genes, we created custom bacterial artificial chromosome (BAC) microarrays containing the genomic sequence at 18q21-18q23. Analysis of 34 prostate tumors using array comparative genomic hybridization revealed gains and losses within this region. Transfection of the metastatic prostate cancer cell line PC-3 with BAC clones containing the maspin and NETO1 genes results in altered <i>in vitro</i> growth properties. <i>In vivo</i> analysis of the metastatic potential of PC-3 cells transfected with maspin reveals that maspin is involved in the seeding of tumor cells to bone. Additionally, we have evidence that increased copy number of the cadherin-7 gene plays a role in prostate cancer tumorigenesis.					
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## Introduction

Previous studies looking at loss of heterozygosity or allelic imbalance (AI) have implicated numerous chromosomal regions as potential locations for tumor suppressor genes involved in prostate cancer. In primary prostate cancer, the most frequent allelic losses occur at 8p, 10q, 11p, 16q, 17p, 18q, and 21q (Cunningham et al., 1996; Saric et al., 1999). In addition, in metastatic prostate cancer specimens there is an increased frequency of AI at 18q compared to primary cancer samples, and there are two distinct regions of loss on 18q associated with the metastatic samples (Padalecki et al., 2000). These data imply that loss of chromosome 18q loci is a metastasis-related event. We have found that the introduction of chromosome 18 into the metastatic human prostate cancer cell line PC-3 caused dramatic phenotypic changes, including a suppression of metastatic growth in an *in vivo* model for metastatic potential. The hypothesis for this study was that introduction of chromosome 18 into the PC-3 cell line complements the distal region of loss on 18q found in both metastatic prostate cancer specimens, and in the PC-3 cell line. The overall objective of this research was to identify the gene(s) on 18q22 responsible for suppressing the potential of PC-3 prostate cancer cells to metastasize to bone.

## Body

The research accomplishments for the final reporting period: 12/1/2001-11/30/2005

### **Task 1: Develop efficient *in vitro* assay to analyze metastatic potential.**

We optimized invasion assays with the PC-3 prostate cancer cells through Matrigel basement membrane matrix (Becton-Dickinson, Bedford, MA) using serum as a chemoattractant. Originally, we anticipated the generation of numerous transfected PC-3 clones containing different regions of chromosome 18q. However, due to the limited number of genomic clones that showed statistically significant alterations (gains or losses) in the comparative genomic hybridization microarray (array CGH) experiments in Task 2, we proceeded directly to analyzing the expression of candidate genes and to the *in vivo* assays using nude mice to characterize any changes in metastatic potential (reported in annual reports 2003 and 2004).

### **Task 2: Narrow the size of the critical region containing the gene involved in metastasis suppression.**

In order to identify a gene on chromosome 18q involved in suppressing metastatic prostate cancer, it was imperative to narrow the sizes of the two critical regions defined using the metastatic prostate cancer specimens. These regions are approximately 7 and 6 centimorgans by genetic markers, respectively. To accomplish this task, we constructed minimum tiling contigs of bacterial artificial chromosomes (BACs) that encompass both the proximal and distal regions of loss on chromosome 18q. We constructed two microarray platforms, one contained only the proximal and distal region, the other platform included the proximal and distal region, plus the intervening sequence (165 BAC clones). Both of these genomic arrays were spotted onto slides by Spectral Genomics (Houston, TX).

We obtained 34 prostate tumors from the UTHSCSA prostate tumor bank directed by Dr. Dean Troyer. DNA was isolated from the tumors, fluorescently-labeled and hybridized to the genomic microarrays. The fluorescent intensity of the spots was analyzed and normalized using SpectralWare software (Spectral Genomics). Using the array comparative genomic

hybridization (CGH) method on two chromosome 18 custom microarrays, we have identified specific regions of deletion in the prostate tumors (Tables 1 and 2), as reported in annual report 2004.

Table 1. List of RP11-BAC clones, cytogenetic position, percentage of deletion in prostate tumor samples, and candidate genes identified by platform 1 array CGH.

<b><u>RP11 BAC Clone</u></b>	<b><u>Position</u></b>	<b><u>% Deleted</u></b>	<b><u>Candidate Genes</u></b>
87B19	49.8-50.0	52.63%	MBD2
61D1	50.2-50.3	52.63%	
450M22	51.7-51.9	36.84%	
49M12	53.4-53.5	42.11%	FECH, NARS, ATP8b1
390D24	54.0-54.2	52.60%	NEDDL4
843G18	54.0-54.2	42.11%	
61J14	54.6-54.7	26.32%	MALT1, ZNF532
890O5	55.0-55.2	31.58%	RAX, CMPLX4, LMAN1
888I12	56.3-56.5	47.37%	
1061K3	68.1-68.2	26.32%	
25L3	69.6-69.8	57.89%	
737F6	69.8-69.9	42.11%	FBX15
1133K3	69.9-70.0	31.58%	FBX15, HSPC154
714E22	69.95-70.1	42.11%	FBX15, CYB5
231E6	70.2-70.4	42.11%	CNDP1, CNDP2

#### **A. RP11-25L3 deletion**

As reported in annual report 2004, in 58% of the tumors we have observed loss of chromosome 18q DNA encompassing the region contained in BAC clone RPCI-11 25L3 (RP11-25L3). We have confirmed the loss of this genomic region in prostate tumors using fluorescence in situ hybridization (FISH) on paraffin-embedded sections (Figure 1). The genomic region contained in the 25L3 clone is homozygously deleted in 58% of the tumors. Additionally, the normal adjacent tissue from the same patients exhibited two copies of this region, indicating that the deletion is tumor-specific (Figure 1). We recently defined the minimal region of loss specifically to the small segment within the RP11-25L3 clone, because the proximal and distal genomic clones flanking RP11-25L3 were present in two copies in 100% (3/3) of prostate tumors analyzed (Figure 1).

Since loss of the region within the RP11-25L3 genomic clone appears to be a frequent event in prostate cancer, we wanted to determine whether the loss is a prostate tumor-specific event or occurs commonly during carcinogenesis of other tissues. Therefore, we performed FISH analysis on tissue microarrays (Imgenex, San Diego, CA) containing tumor specimens from liver, bladder, pancreas, ovary, breast, and prostate. Both breast and prostate tumor specimens were homozygously-deleted for the RP11-25L3 genomic clone. Cancerous tissues from the liver, bladder, pancreas, and ovary contained two copies of RP11-25L3. Of the breast

tumors analyzed, 50% (5/10) contained a homozygous deletion of the RP11-25L3 genomic clone. However, the chromosome 18q content within the ten cells counted per breast cancer specimen was variable. That is, some cells lost an entire chromosome 18 and retained one copy of 18 including RP11-25L3. Some cells appeared homozygously deleted for RP11-25L3, whereas other cells within the same tumor had two copies of RP11-25L3. For example, in breast cancer specimen six, four cells were homozygously deleted for RP11-25L3 and six cells were normal. Of the breast cancer specimens that exhibited a deletion of the RP11-25L3 genomic segment, 5/7 (71%) were associated with advanced disease (stage III). Analysis of the prostate tumor tissue revealed a homozygous deletion of the region within the RP11-25L3 genomic clone in 100% (8/8) of the cases. In two of the prostate cancer specimens, there was loss of an entire chromosome 18 and deletion of the region contained within RP11-25L3 on the remaining chromosome.

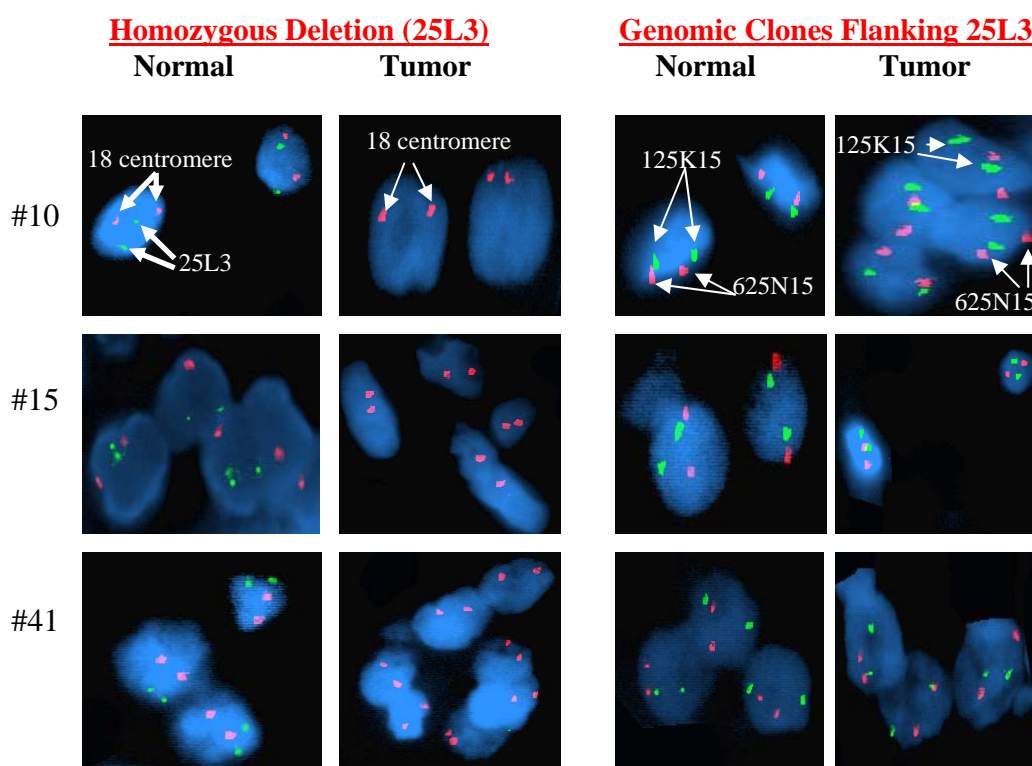


Figure 1: Homozygous deletion of chromosome 18 DNA contained with RP11-25L3.

Prostate tumor specimens #10, #15, and #41 exhibit loss of both copies of the region contained within RP11-25L3 (green) while retaining two copies of the chromosome 18 centromere (red). Normal adjacent prostate tissue contains two copies of the region contained within RP11-25L3 and the chromosome 18 centromere. In order to define the extent of the homozygous deletion, the genomic clones flanking RP11-25L3 were analyzed in prostate tumor specimens #10, #15, and #41. The proximal clone RP11-126K15 (green) and distal clone RP11-625N15 (red) are retained as two copies in the normal adjacent prostate tissue, as well as in the prostate tumor.

There are no identified genes associated with the genomic region contained in the RP11-25L3 clone. Using SAGE (serial analysis of gene expression) tag analyses, Chen et al. (2002) were able to demonstrate that 67% of unmatched SAGE tags in the SAGE database are derived from novel transcripts and may represent unidentified novel genes. Additionally, the number of genes and transcripts may be grossly underestimated based on SAGE analyses. The specific loss of this defined segment in breast and prostate tumor strongly implies that the region may contain an unidentified tumor suppressor gene or noncoding regulatory sequences such as microRNAs (miRNAs), directly or indirectly involved in tumorigenesis.

### **B. Maspin deletion**

Using the second platform consisting of array CGH slides providing genomic coverage of the entire 18q21-q23 region, we have also observed the deletion of the region containing the maspin gene in 19% of prostate tumors (annual report 2004; Table 2). Maspin is a member of the serine protease inhibitor family and has been found to be down-regulated in breast carcinoma (Zou et al., 1994). Loss of maspin expression has also been implicated in prostate cancer, as described by Cher et al. (2003), who transfected the prostate cancer cell line DU145 (derived from a brain metastasis) with a high-level expression construct for maspin. The effects of transfecting maspin into DU145 cells, as analyzed with an *in vivo* model for bone matrix remodeling and tumor growth were decreased tumor growth, reduced osteolysis, and decreased angiogenesis (Cher et al., 2003).

Table 2. List of RP11-BAC clones, cytogenetic position, percentage deleted in prostate tumor samples, and candidate genes identified on the second platform using array CGH. (Highlighted regions are continuous stretches of deleted BAC clones).

<b>RP11-BAC Clone</b>	<b>Position</b>	<b>% Deleted</b>	<b>Genes</b>
87B19	49.5-50	29%	MBD2
<b>275K5</b>	<b>53.5-53.7</b>	<b>24%</b>	<b>ATP8B1</b>
<b>693L9</b>	<b>53.7-53.9</b>	<b>29%</b>	<b>NO KNOWN GENES</b>
<b>718I15</b>	<b>53.8-54</b>	<b>19%</b>	<b>NEDD4L</b>
<b>108P20</b>	<b>54.5-54.7</b>	<b>24%</b>	<b>MALT1</b>
<b>675P24</b>	<b>54.6-54.8</b>	<b>24%</b>	<b>ZNF532</b>
<b>685A21</b>	<b>54.7-54.9</b>	<b>14%</b>	<b>ZNF532</b>
<b>350K6</b>	<b>54.8-55</b>	<b>19%</b>	<b>SEC11L3</b>
<b>569D6</b>	<b>54.9-55.1</b>	<b>24%</b>	<b>SEC11L3, GRP, RAX, CPLX4</b>
<b>760L24</b>	<b>55.3-55.5</b>	<b>29%</b>	<b>CCBE</b>
<b>2N1</b>	<b>55.3-55.5</b>	<b>24%</b>	<b>CCBE</b>
<b>824M15</b>	<b>55.5-55.7</b>	<b>38%</b>	<b>CCBE, RPS3A</b>
<b>103A19</b>	<b>55.6-55.8</b>	<b>29%</b>	<b>GDNF</b>
<b>120K19</b>	<b>58.3-58.4</b>	<b>19%</b>	<b>ZCCHC2</b>
<b>13L22</b>	<b>58.4-58.6</b>	<b>19%</b>	<b>PHLPP</b>
<b>635N19</b>	<b>59.2-59.3</b>	<b>19%</b>	<b>MASPIN</b>
<b>41O4</b>	<b>66.1-66.2</b>	<b>19%</b>	<b>SOCS6</b>
<b>231E4</b>	<b>70.2-70.4</b>	<b>19%</b>	<b>CNDP1, CNDP2</b>

### C. NETO1 deletion

In one prostate tumor, we observed deletion of the region contained in the BAC clone RP11-676J15. The gene neuropilin and tolloid-like protein 1 (NETO1), located within the distal region of loss at 18q22.3, is encoded in this region. NETO1 encodes a putative type I transmembrane protein consisting of two extracellular CUB domains (named derived from the complement subcomponents C1r/C1s, an embryonic sea urchin protein uEGF and bone morphogenic protein-1), a N-terminal signal sequence, low-density lipoprotein receptor class A module, and a conserved cytoplasmic FXNPXY-like motif (Stohr et al., 2002). The CUB motifs in the NETO1 protein share homology to those found in neuropilins and the *Drosophila* dorsal-ventral patterning protein tolloid. Due to this similarity, NETO1 may be involved in neuronal development and/or VEGF signaling. Further analysis of this gene is described under Task 3.

### D. Cadherin-7 amplification

As described in annual report 2004, we also discovered a small region of amplification in the chromosome 18 genome, using the platform 2 microarray, between the proximal and distal regions of AI. Array CGH results from 22 primary prostate tumors has revealed a discrete amplification of chromosome 18q22.1 in 21/22 tumors. Of the 22 tumors, nine exhibited a low level of amplification, nine exhibited a medium level of amplification and 3 tumors were highly-amplified in this region. Only one tumor appeared to have the normal two copies of this region. In two tumors, the amplification appeared limited to a very small region encompassing the genomic DNA contained in BAC clone RP11-775G23 (Figure 2). The other 19 tumors exhibited amplification of a larger region encompassing RP11-775G23, but also extending beyond approximately 2 megabases. The BAC clone RP11-775G23 encodes a portion of the cadherin-7 gene (CDH7) the remainder of the CDH7 gene is encoded in the DNA contained in BAC clone RP11-389J22. Interestingly, the gene for cadherin-19 (CDH19) is located only 800 kilobases away. In addition, the gene for cadherin-20 (CDH20) is located 5 megabases proximal to CDH7. FISH analyses on paraffin-embedded section using CDH7 genomic probes confirmed the amplification of this region (Figure 3). These were exciting novel results as chromosome 18 gene amplification has never previously been associated with prostate cancer. Cadherin-7, -19 and -20 are atypical type II cadherins (mesenchymal-type) and altered expression of type II cadherins in breast cancer has been shown to be associated with invasive breast cancer (Feldes et al., 2002) (Manuscript describing this work included in appendix).

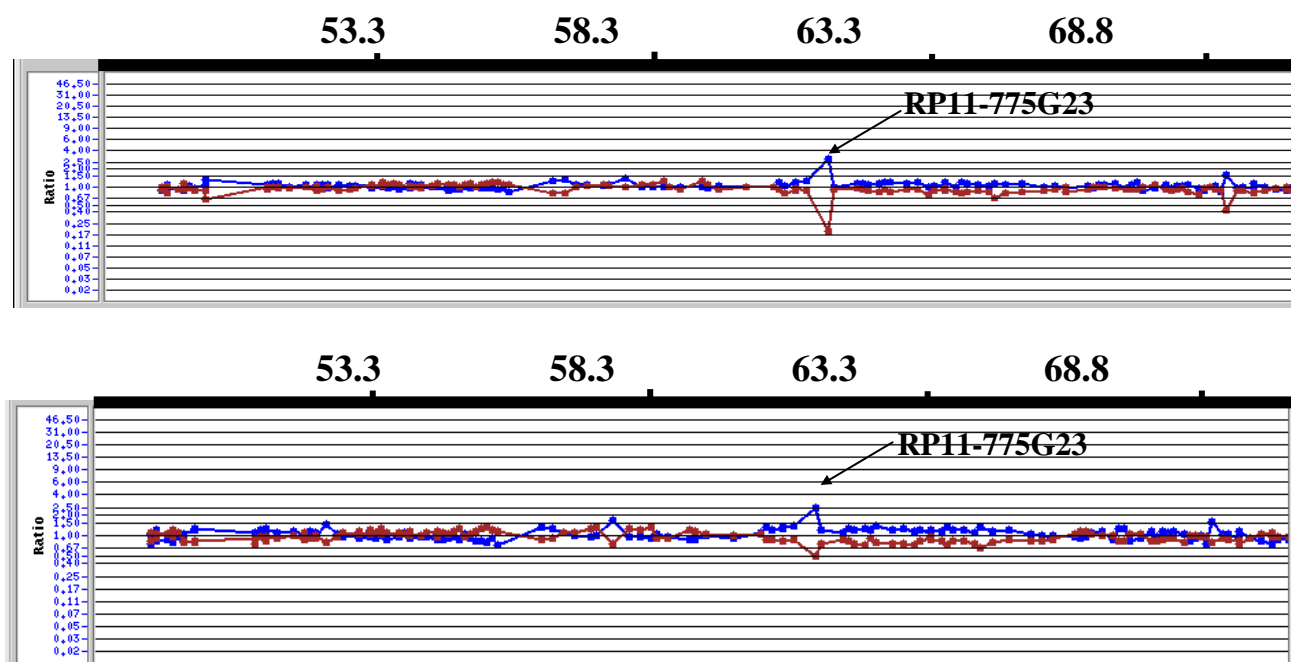


Figure 2: Ratio plots from chromosome 18 array CGH for prostate tumors N40 and N41.

The plots comprise normalized data from two independent array experiments. In one array, the genomic DNAs from tumor samples were labeled with Cy3 (red) and the male reference DNA labeled with Cy5 (blue). In the other array, the tumor sample DNAs were labeled with Cy5 (blue) and the reference DNA with Cy3 (red) in dye swap experiments. The fluorescent signals were analyzed and normalized Cy5:Cy3 ratios were computed for both arrays using SpectralWare software (Spectral Genomics, Houston, TX). On the ratio plot, blue in the positive deviation (upwards) depicts amplification. The X-axis represents the cytogenetic position of each BAC clone (in megabases) and the Y-axis represents the log<sub>2</sub> ratio of Cy5/Cy3.

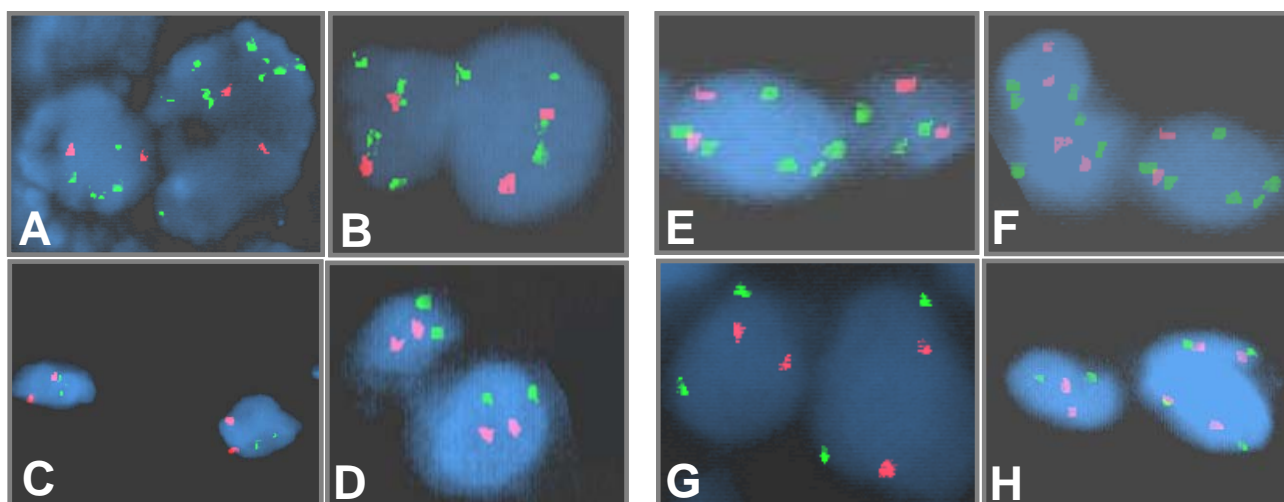


Figure 3: Validation of the amplification at chromosome 18q22.1 using FISH on paraffin-embedded tumor sections.

A probe derived from BAC clone RP11-775G23 (FITC-green) detects multiple copies of this region in the prostate tumors (**A and B**). Corresponding adjacent normal cells show only two copies of this region (**C and D**). The control probe (spectrum orange-red) was a human chromosome 18-specific centromeric probe. A probe derived from RP11-389J22, the clone flanking RP11-775G23, also shows amplification in the tumors (**E and F**), while adjacent normal cells (**G and H**) show only two copies. Both clones RP11-775G23 and RP11-389J22 contain portions of the CDH7 gene.

### Task 3: Introduce BACs from the region into prostate cancer cells and analyze the phenotype.

In order to introduce BAC clones into prostate cancer cells and have the DNA be selectively retained, it is necessary to introduce a selectable marker into the BAC vector. We obtained the targeting vector pRetroES (Wang et al., 2001) from American Type Tissue Collection, which enabled us to retrofit the BAC vectors with a mammalian selectable marker. This permits cells containing the BAC clone to selectively grow in tissue culture medium containing G418. This is a very efficient procedure that enabled us to retrofit BAC clones RP11-635N19 (maspin) and RP11-676J15 (NETO1), with no resulting deletions or recombinations.

#### A. Maspin

Using array CGH, we identified a region of loss containing the gene for maspin, as described in the annual report 2004. Quantitative RT-PCR analyses revealed that maspin is expressed abundantly in prostate epithelial cell lines, but maspin mRNA is either absent or significantly reduced in all prostate cancer cell lines analyzed. Array CGH analysis of 21 prostate tumor samples revealed that the region of chromosome 18 containing the maspin gene is deleted in 19% of cases. Since the most common site for metastasis of prostate cancer cells is bone, we utilized the PC-3 prostate cancer cell line, which was originally derived from a bone metastasis, to assess the ability of maspin to suppress bone metastasis in an *in vivo* model.

Quantitative RT-PCR analysis of maspin mRNA revealed that the PC-3 prostate cancer cell line had a 4.7-fold reduction in maspin mRNA levels, compared to prostate epithelial cell lines. Previous *in vivo* metastasis studies by our lab showed that the PC-3 cell line consistently metastasized to bone and this metastatic potential was significantly reduced following introduction of an intact human chromosome 18 (Padalecki et al., 2003, appendix). The serine protease inhibitor maspin has been implicated in prostate cancer progression and is a candidate gene for the reduction of metastasis to bone following introduction of chromosome 18 into the PC-3 prostate cancer cell line.

In order to determine whether maspin expression accounted for the inhibition of tumor growth and metastasis observed after introduction of an intact 18 into PC-3 cells, we transfected the maspin gene into PC-3 cells. In an effort to recreate the physiological expression levels of maspin under the control of the endogenous maspin promoter, we transfected the entire BAC clone containing the maspin gene (RP11-635N19) into the PC-3 cell line. This experiment was novel in that the previous study analyzing the effect of maspin on the ability of a prostate cancer cell line (DU145) to metastasize to bone utilized a high-level expression vector which overexpressed maspin, compared to the levels normally found in prostate epithelial cells (Cher et al., 2003). Using a BAC retrofitting approach, we inserted the neomycin-resistance selectable marker into the BAC vector allowing for a more efficient transfection and preserving the integrity of our inserted genomic fragment. Following transfection, isolation, and expansion of individual clones in medium containing G418, PC-3 maspin transfectants were analyzed for expression of the maspin gene by quantitative RT-PCR analysis. Two of the PC-3 maspin clones (B7 and C5) had increased expression of the maspin gene compared to parental PC-3 cell line. The PC-3 maspin B7 and C5 clones demonstrated 1.7- and 3.0-fold increased expression, respectively, compared to the parental cell line PC-3.

To determine whether increased expression of the maspin gene affected the *in vitro* growth characteristics of PC-3 cells, we assessed the doubling time and anchorage independent growth of the maspin transfectants, compared to the parental cell line. The PC-3 maspin clones B7 and C5 exhibited an increase in doubling time, compared to the parental cell line. The doubling times of clones PC-3 maspin B7 and C5 were approximately 48 hr and 40 hr respectively, compared to 21 hr in the PC-3 cells. Anchorage-independent growth is a hallmark of the transformed phenotype of cancer cells. The PC-3 maspin clones B7 and C5 were unable to form colonies in soft agar, compared to the high-efficiency of PC-3 cells to form colonies. The results of the doubling time and soft agar experiments imply that expression of maspin alters the *in vitro* growth characteristics of prostate cancer cells.

Since the primary site of metastasis in prostate cancer patients is bone, we performed an *in vivo* metastasis assay using the PC-3 maspin transfectants. Previous experiments in our lab have demonstrated that PC-3 cells consistently metastasize to bone (Padalecki et al., 2003, appendix). In an *in vivo* metastasis model, PC-3 and PC-3 maspin transfectants were injected into the left cardiac ventricle of individual athymic nude mice (10 mice per cell line) and monitored by radiography on a regular basis, to assess the effect of maspin on the metastatic potential of PC-3 cells. The animals injected with the parental PC-3 cells developed debilitating bone lesions and had to be sacrificed at six weeks. Animals injected with the PC-3 maspin clones B7 and C5, as well as a PC-3 microcell hybrid containing an exogenously introduced intact chromosome 18, had few if any bone lesions and none were as dramatic as those seen in animals injected with the parental PC-3 cells. After following the mice for 18 weeks, which compensated for the reduced doubling time of the PC-3 maspin transfectant clones compared to

PC-3 parental cells, the remaining mice were sacrificed and the bones prepared for analyses. By quantitative histomorphometry, the PC-3 maspin transfectants exhibited significantly fewer bone metastases, compared to the PC-3 parental cell line. Quantitative radiographic analysis revealed that animals injected with the PC-3 maspin transfectants and a chromosome 18-containing PC-3 hybrid showed a significant reduction in the number of bone lesions (Figures 4 and 5). However, the numbers of soft tissue metastases were similar in number between the PC-3 parental control and the PC-3 cells with the introduced maspin gene. These data imply that maspin may not be involved in the general process of metastasis, but may instead play a specific role in the ability of the PC-3 prostate cancer cells to seed to bone.

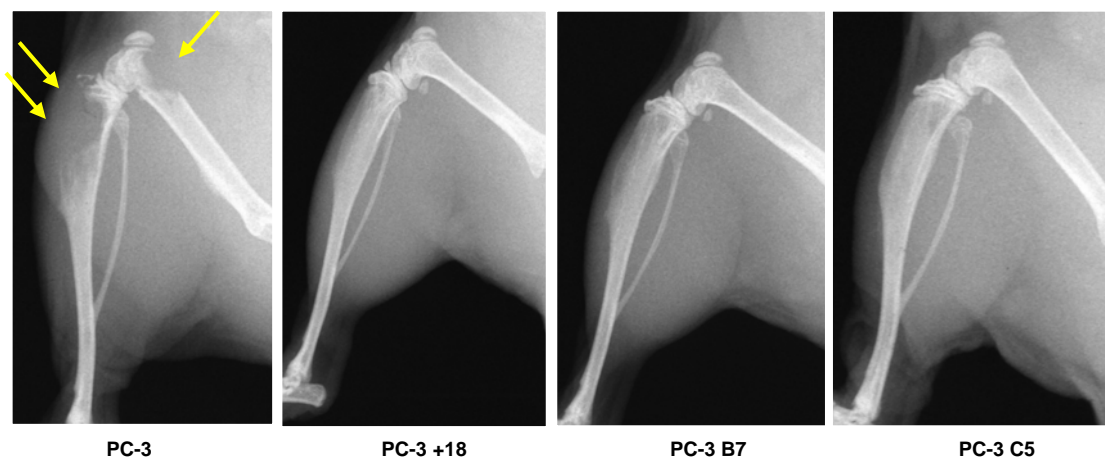


Figure 4: Radiographic images of *in vivo* metastasis assay

PC-3 maspin transfectant clones B7 and C5 and PC-3 microcell hybrids containing an intact chromosome 18 exhibit reduced metastasis to bone. The arrows denote osteolytic bone lesions due to metastasis of parental cell line PC-3.

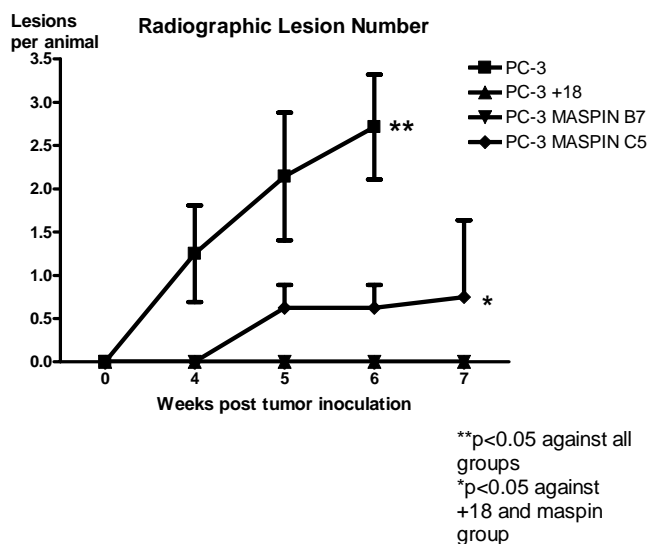


Figure 5: Number of metastatic bone lesions as determined by quantitative radiography.

PC-3 maspin transfectants show reduced metastasis to bone.

## B. NETO1

In an effort to localize a putative tumor suppressor gene involved in prostate cancer progression, expression analysis studies were performed on the 27 known genes within the two proximal and distal critical regions of loss. Only one particular gene neuropilin and tolloid-like protein 1 (NETO1), located within the distal region of loss (18q22.3) in prostate cancer cell lines, had an expression pattern consistent with a tumor suppressor gene, which was described in annual report 2004. NETO1 was expressed in prostate epithelial cells and had absent or reduced expression in prostate cancer cell lines. NETO1 encodes a putative type I transmembrane protein consisting of two extracellular CUB domains (Stohr et al., 2002). The CUB motifs in the NETO1 protein share homology to those found in neuropilins and the *Drosophila* dorsal-ventral patterning protein tolloid. Due to this similarity, NETO1 may be involved in neuronal development and/or VEGF signaling.

The undetectable levels of mRNA of the NETO1 gene in the DU145 prostate cancer cell line was confirmed by quantitative RT-PCR analysis, which also revealed decreased expression in PC-3 and 22Rv1 prostate cancer cell lines. However, elevated expression of NETO1 was observed in the human prostate cancer cell line LNCaP. Since NETO1 exhibited reduced expression levels in three different prostate cancer cell lines, we analyzed the level of NETO1 expression in several human prostate tumor specimens. Although the levels of the NETO1 mRNA were decreased in some prostate tumors, expression was increased in others.

At the genomic level, the BAC clone RP11-676J15, containing the NETO1 gene, was used as a probe for paraffin-embedded FISH analysis to determine the copy number of NETO1 in prostate tumors. Our recent FISH analysis revealed an unstable genomic pattern in which some tumor cells contained two, one, or no copies of NETO1 (Figure 6). Overall, the majority of cells analyzed had only one copy of the NETO1 gene.

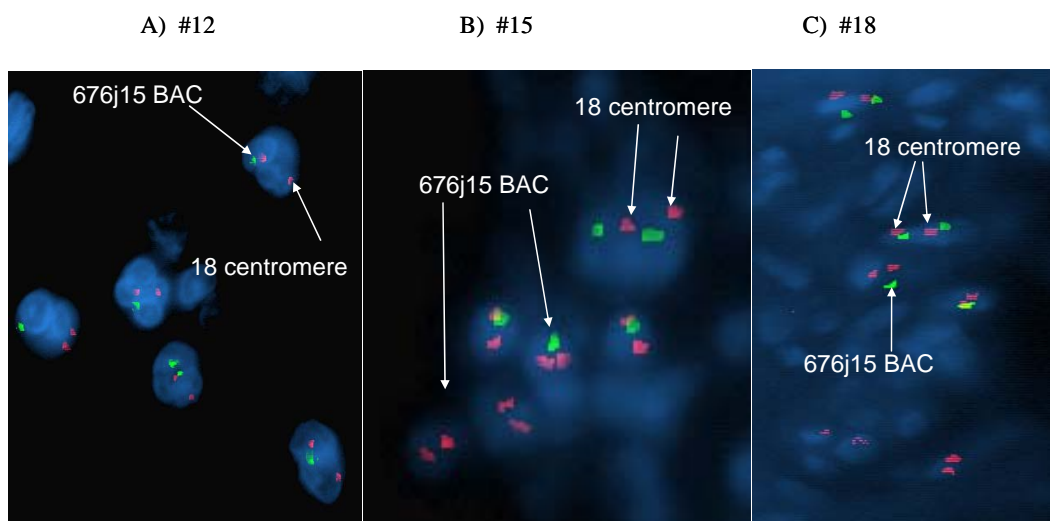


Figure 6: FISH results from paraffin-embedded prostate tumor samples.

Prostate tumor specimens #12, #15, and #18 exhibit loss of either one or two copies of the region contained within BAC RP11-676J15 (NETO1) (green), while retaining two copies of the chromosome 18 centromeric probe (red). Normal adjacent prostate tissue has the normal two copies of the region contained within RP11-676J15 and the chromosome 18 centromere.

Since NETO1 expression was absent in the prostate cancer cell line DU145, we introduced the NETO1 gene into DU145 cells to determine if expression of NETO1 would rescue the cancerous phenotype. In order to express NETO1 under the control of the endogenous promoter, we transfected a genomic clone containing the NETO1 gene into the DU145 prostate cancer cell line. By using a genomic clone, expression of the NETO1 gene was regulated by a natural promoter and the mRNA levels would be at a more physiological level. Following transfection, isolation, and expansion of individual clones, DU145 NETO1 transfectants were analyzed for expression of the NETO1 gene by quantitative RT-PCR analysis. Of the 21 DU145 NETO1 clones isolated, none expressed NETO1.

Subsequently, we introduced the NETO1 cDNA into the DU145 prostate cancer cell line using an expression construct driven by a strong promoter. Under these conditions, transfection of DU145 cells with the NETO1 cDNA resulted in the initial growth of numerous colonies. However, none of these colonies grew to a large enough size to be clonally isolated. The mock-transfected (vector only) DU145 cells grew rapidly and could be clonally isolated and expanded. It appears the expression of NETO1 in DU145 cells dramatically affected their growth. These results could be interpreted to mean that NETO1 may potentially play a role in cellular growth regulation. The effects of NETO1 on cancer cell growth may involve the interaction of NETO1 with neuropilins, semaphorins, and VEGF. More experiments must be done to investigate the function of NETO1 in prostate cells.

#### **Task 4: Analyze the DNA sequence from the genomic clones for open reading frames (genes).**

With the data available from the Human Genome Project, we have identified known genes and expressed sequence tagged sites (ESTs) from both the proximal and distal region of loss in metastatic prostate tumors. As described in annual report 2004, we previously designed primer sets for these 24 genes/ESTs and analyzed the expression levels of these genes/ESTs by reverse transcription/PCR in a normal prostate epithelial line and in six commercially-available metastatic prostate cancer cell lines to identify candidate tumor suppressor genes.

Of all the genes/ESTs analyzed, only the NETO1 gene, located in the distal region, had an expression pattern that would be suggestive of a putative tumor suppressor gene. NETO1 was expressed in prostate epithelial cells, but exhibited reduced expression in prostate cancer cell lines and tumor specimens. As described in Task 3, NETO1 is in one copy in some of the tumor cells and in two copies in other tumors cells within the same prostate tumor. Expression of NETO1 does appear to alter the in vitro growth phenotype of a prostate cancer cell line. More experiments are ongoing to better define the function of NETO1 in prostate cells.

We identified the homozygous deletion of the chromosome 18 genome contained within the BAC clone RP11-25L3 in prostate and breast cancer samples. We analyzed the clone for open reading frames and searched against databases of known miRNAs, but did not identify any coding sequences or noncoding regulatory sequences. Since this deletion appears to be specific to prostate and breast cancer samples, it is highly probable that this region contains a, as yet unidentified, noncoding regulatory sequence (miRNA) or a gene. Further experiments are being conducted to determine if the same deletion breakpoints occur in all the tumors and whether introduction of the RP11-25L3 BAC clone into prostate cancer cell lines alters the cellular phenotype.

## Key Research Accomplishments

- Performed hybridizations with DNA isolated from prostate cancer specimens/cell lines onto chromosome 18 microarray slides and identified specific regions which show reduced copy number in prostate cancer.
- Identified a region of amplification of chromosome 18 containing two type II cadherin genes in prostate cancer specimens.
- Transfected genomic clones corresponding to the NETO1 and maspin genes into prostate cancer cell lines and observed dramatic changes in growth *in vitro*. Completed *in vivo* experiments analyzing the metastatic potential of PC-3 cells transfected with the maspin genomic clone and demonstrated that expression of maspin at endogenous levels affects the potential of PC-3 to seed to bone.
- Abstract presented at the 2005 American Association for Cancer Research meeting Hall, D.C., Vijayakumar, S., Naylor, S., Troyer, D.A., Reveles, X.T., **Johnson-Pais, T.L.**, Leach R.J. 2005. Array comparative genomic hybridization analyses of chromosome 18q in prostate tumor samples. Proc Amer Assoc Cancer Res **46**:3578.
- Three manuscripts describing studies of 1) cadherin amplification; 2) the role of maspin in the metastasis of prostate cancer cells to bone; and 3) the effect of NETO1 expression on the growth of prostate cancer cells, are in the process of submission.

## Reportable outcomes

- Published manuscript:  
Padalecki SS, Weldon KS, Reveles XT, Buller CL, Grubbs B, Cui Y, Yin JJ, Hall DC, Hummer BT, Weissman BE, Dallas M, Guise TA, Leach RJ, Johnson-Pais TL 2003. Chromosome 18 suppresses prostate cancer metastases. Urol Oncol 21:366-373.
- A portion of this research was presented as a poster at the 2005 American Association for Cancer Research 2005 Annual Meeting.
- This grant supported the training of Ph.D. student Devon Hall, who will defend his dissertation research in January 2006.
- I was recently awarded a 2005 CDMRP US DOD Prostate cancer research program Idea Development Award entitled "Amplification of Type II Cadherins in Prostate Cancer" contract # W81XWH-06-1-0090 to investigate the role of cadherins 7 and 19 amplification in prostate cancer, using preliminary data generated from this New Investigator Award.

## Conclusions

We have generated data showing gains and losses of chromosome 18q in prostate cancer. The microarray experiments generated more data than the previous allelic imbalance experiments because the samples did not have to be heterozygous for 18q markers. Allelic imbalance experiments are also not useful for the identification of amplifications, which we have now discovered in the 18q22.1 region. Since the prostate specific antigen test is such a poor tool for the identification and characterization of aggressive prostate cancer, the identification of genes whose loss or gain could serve as molecular markers to identify highly aggressive prostate cancer which would be an extremely useful clinical tool. The data generated from these tasks, including the discovery of the cadherin-7 amplification in prostate cancer specimens and the breast and prostate cancer-specific deletion of the genome contained in the RP11-25L3 clone have provided us with preliminary data that has the potential of being developed in a prostate cancer biomarker. Future experiments are being designed to define the exact region of homozygous deletion in prostate tumors, which encompasses the RP11-25L3 region. We are also in the process of creating polyclonal antibodies to determine the cadherin-7 protein levels in prostate tumors of various stages and grades to determine if the increased gene copy number correlates with increased protein expression.

## Bibliography from research effort

### Abstract:

Hall, D.C., Vijayakumar, S., Naylor, S., Troyer, D.A., Reveles, X.T., **Johnson-Pais, T.L.**, Leach R.J. 2005. Array comparative genomic hybridization analyses of chromosome 18q in prostate tumor samples. *Proc Amer Assoc Cancer Res* **46**:3578.

### Publication:

Padalecki SS, Weldon KS, Reveles XT, Buller CL, Grubbs B, Cui Y, Yin JJ, Hall DC, Hummer BT, Weissman BE, Dallas M, Guise TA, Leach RJ, Johnson-Pais TL 2003. Chromosome 18 suppresses prostate cancer metastases. *Urol Oncol* 21:366-373.

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## Appendix:

### Original article

## Chromosome 18 suppresses prostate cancer metastases

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## Abstract

Loss of heterozygosity and allelic imbalance data has shown that there are two distinct regions of loss on chromosome 18q associated with the progression of prostate cancer (CaP). To investigate the functional significance of chromosome 18q loci in CaP, we utilized the technique of microcell-mediated chromosome transfer to introduce an intact chromosome 18 into the human prostate cancer cell line, PC-3. Three of the resulting hybrid lines were compared to the PC-3 cells in vitro and in vivo. The hybrid cell lines, containing an intact copy of the introduced chromosome 18, exhibited a substantial reduction in anchorage-dependent and independent growth in vitro. These hybrid cell lines also made smaller tumors in nude mice following subcutaneous injection compared to PC-3 cells. Because tumor growth was not completely eliminated by introduction of chromosome 18, we assessed the ability of the hybrids to metastasize to bone after intra-cardiac inoculation in a nude mouse model. Mice inoculated with PC-3 hybrids containing intact copies of chromosome 18 had significantly fewer bone metastases and dramatically improved survival compared to PC-3 cells. In addition, the introduction of chromosome 18 significantly reduced tumor burden in extraskelatal sites. This was not because of differences in growth rates because mice bearing hybrids were monitored for metastases over twice as long as mice bearing PC-3 cells. Taken together, these data suggest that chromosome 18

has a functional role in CaP to suppress growth and metastases. Identification of the responsible gene(s) may lead to molecular targets for drug discovery.

**Author Keywords:** Prostate cancer; Chromosome 18; Metastasis

## 1. Introduction

Prostate cancer (CaP) is the most common form of noncutaneous malignancy in American men. More than 198,000 men were diagnosed in 2001 and greater than 31,500 died (Cancer Facts and Figures 2001, American Cancer Society; <http://www3.cancer.org/cancerinfo>). While more cases are diagnosed at early stage, there is no accurate way to determine which prostate cancers have metastatic potential that would benefit from immediate intervention. CaP results from the accumulation of genetic lesions, which leads to tumor development and progression. One area of CaP research has identified consistent regions of allelic imbalance as an indication of genetic alterations in cancer-related genes such as oncogenes, tumor suppressor, metastasis suppressor, and DNA repair genes. Molecular studies of CaP have identified multiple nonrandom genetic alterations in tumor specimens. Our recent AI study presents evidence for two independent loci on chromosome 18q involved in the progression of CaP [1]. To date, no known tumor suppressor genes or metastasis suppressor genes have been identified in these regions of chromosome 18.

We also examined CaP cell lines to determine if they harbored extended regions of homozygosity on chromosome 18, which imply the loss of a tumor suppressor gene. The CaP cell line PC-3 exhibited a long stretch of homozygosity between markers D18S1091 and D18S469, that overlapped with the distal region of loss identified in metastatic patient samples [1]. Thus, PC-3 is an excellent cell line in which to test the functional significance of loss in this region.

To establish a functional assay to examine the effects of chromosome 18 on CaP cells, we utilized microcell-mediated chromosome transfer (MMCT), which introduces a selectively marked chromosome into a cell line of choice. The resulting microcell hybrids can be assessed for the capacity of the introduced chromosome to suppress the tumorigenic properties of the original CaP cell line.

This technique has been utilized in the past to demonstrate the existence of tumor suppressor genes on other chromosomes in CaP. Ewing et al. [2] provided evidence that chromosome 5 suppressed the tumorigenic phenotype of PC-3. Other laboratories have shown tumor suppressor functions attributable to chromosomes 10p [3, 4 and 5], 17q [6] in the CaP cell line PC-3, and the related line, PPC-1. Numerous investigators have provided evidence of metastasis-suppressor function via MMCT to introduce human chromosomes of interest into Dunning rat CaP cell lines [7, 8, 9, 10, 11 and 12].

Introduction of a normal copy of human chromosome 18 into other human cell lines has yielded similar results. Chromosome 18 suppressed the tumorigenicity of the human colon carcinoma cell line, COKFu [13], as well as colorectal cancer cells, SW480 [14] and endometrial carcinoma lines HHUA and Ishikawa [15]. Recently, we reported evidence supporting the existence of a tumor suppressor gene on chromosome 18 between the genetic markers, D18S848 and D18S58,

by complementation of this region in both the CaP cell line, DU-145 and TSU-PR1 cells [16]. The latter cell line was recently shown to be bladder cancer rather than CaP in origin [17]. These experiments further suggest that putative tumor suppressor gene(s) on chromosome 18 have the potential to play a role in a variety of tumor types.

Here, we report that introducing an intact copy of chromosome 18 into the metastatic CaP cell line, PC-3, suppresses metastases. Our results support AI or loss of heterozygosity (LOH) data that implicate chromosome 18 as the site of a tumor suppressor gene or metastasis suppressor gene involved in CaP. Furthermore, they provide the first evidence of metastasis suppression by chromosome 18 in an in vivo model of human CaP.

## **2. Materials and methods**

### **2.1. Cell lines**

The cell line, PC-3 (obtained from American Type Culture Collection, Rockville, MD), was used as the recipient in the MMCT experiments. PC-3 was established from a human CaP adenocarcinoma to bone [18]. It is both tumorigenic and metastatic in athymic nude mice [18, 19, 20, 21 and 22]. It forms primarily osteolytic skeletal metastases as well as extraskelatal metastases following intra-cardiac inoculation of tumor cells into nude mice.

The cell lines, A9.18neo [13] (kindly provided by Dr. J. Carl Barrett) and MCH912.1 [23] (kindly provided by Dr. Eric Stanbridge) were used as donors in the MMCT experiments. Both cell lines contain a neomycin-resistance tagged copy of human chromosome 18 in a rodent background.

### **2.2. Molecular characterization of parental cell lines and microcell hybrids**

DNA from the parental cell line and resulting microcell hybrids were analyzed with highly polymorphic microsatellite markers localized to chromosome 18 as previously described [16]. Results from this polymerase chain reaction (PCR)-based analysis were used to identify the number of alleles present in PC-3 as well as the presence of the introduced chromosome in the microcell hybrids. Because normal tissue was not available for the parental CaP cell line, we searched for long stretches of homozygosity that would be statistically unlikely to occur unless there had been an LOH event.

### **2.3. Microcell-mediated chromosome transfer**

For the PC(18)H hybrids, A9.18neo cells containing an integrated neomycin resistance gene were used as donor cells. MMCT experiments were performed as previously described [16]. For the HMC-175 set of hybrids, MMCT experiments were performed as previously described using MCH912.1 cells as the donor cell line [24].

## **2.4. Fluorescence in situ hybridization (FISH)**

Metaphase chromosome spreads of A9.18neo cells were harvested and slides were prepared for FISH analysis with a chromosome 18 paint probe (Vysis, Downers Grove, IL), as previously described [16].

## **2.5. Giemsa-banding of chromosomes**

G-banding of metaphase chromosomes from A9.18neo cells was performed as previously described [16].

## **2.6. In vitro growth**

Cells were plated at  $1 \times 10^4$  cells/ml in 24 well plates at 1 mL per well containing media without selective agents. Three wells were harvested daily for 4–5 days. Cell number was determined using a hemacytometer.

## **2.7. Growth in soft agar**

Cells were plated in agar (top layer: 2.5 mL, 0.24% noble agar (Difco); bottom layer: 5 mL 0.4% agar; both in fully supplemented 1X Dulbecco's Minimal Essential Media) in 60mm tissue culture dishes. Dishes were fed at weekly intervals with 1–2 mL of 1X fully supplemented media. Colonies were scored after 21 days in culture using the advanced colony counting function of the GelExpert97 program on a Nucleovision personal computer system (Nucleotech Corporation, San Mateo, CA). The values are the means of 10 dishes.

## **2.8. In vivo analyses**

All in vivo experiments were performed with the approval of the University of Texas Health Science Center at San Antonio Institutional Animal Care and Use Committee.

## **2.9. Tumorigenicity analysis in nude mice**

The PC-3 parental cell line and PC(18)H and HMC175 hybrid cell lines were inoculated subcutaneously with  $5 \times 10^5$  cells in a 100  $\mu$ L volume into each of five 5- to 7-week-old athymic nude mice (Audie Murphy Veteran's Administration Hospital Nude Mouse Colony, San Antonio, TX). Tumor volumes were monitored every other day using caliper measurements. Tumor volumes were calculated using the following formula for the volume of a sphere,  $V = (\pi/6)d^3$ , where  $V$  = volume and  $D$  = diameter.

## **2.10. Metastasis analysis in athymic nude mice**

Ten athymic nude mice (Audie Murphy Veteran's Administration Hospital Nude Mouse Colony) were inoculated via the left cardiac ventricle with the parental PC-3 cell line or PC-3 hybrids containing an intact copy of the introduced chromosome 18 ( $10^5$  cells/100  $\mu$ L PBS) as previously

described [25]. Mice were monitored daily and followed by radiography to detect skeletal metastases. Body weights were monitored on a weekly basis. Radiographs, in which the mice were prone against the film, were taken at the time of inoculation, and then every 2 weeks until sacrifice. The mice were sacrificed when they developed significant metastases. In the case of PC-3 cells this was on average 2 months after tumor inoculation. Mice inoculated with hybrid cell lines that did not develop significant metastases were sacrificed at 8 months post tumor inoculation to account for the increased doubling time observed in these cell lines. All bones and soft tissues were harvested and fixed in formalin for histological analysis. Necropsy was performed on all mice and those with chest tumors were excluded from the analysis, because this indicated that the tumor inoculum did not properly enter the left cardiac ventricle. All radiographs were evaluated without knowledge of treatment groups. All procedures in the metastasis assay were performed with the approval of our Institutional Animal Care and Use Committee.

### 3. Results

First, we examined the CaP cell line, PC-3, for extended stretches of homozygosity on chromosome 18 using highly polymorphic markers specific for the long arm of chromosome 18. Our previous work implicated chromosome 18 as the location of one or more tumor suppressor genes in CaP [1]. Analysis of PC-3 cells revealed a region of extended homozygosity on the long arm of chromosome 18. This region at 18q22.3 ( $P < 0.005$ ) coincides with a previously identified region of LOH between the markers D18S848 and D18S58 in metastatic CaP specimens [1]. The remainder of the long arm of chromosome 18 was heterozygous by PCR analysis.

To identify a functional role for chromosome 18 in CaP, microcell hybrids were generated by MMCT. Because we sought PC-3 hybrids containing intact copies of chromosome 18, hybrid DNA was analyzed by PCR with 17 highly polymorphic microsatellite markers localized to chromosome 18. This allowed us to examine DNA from these hybrids for the presence of the exogenous copy of chromosome 18. Unfortunately, after our initial experiments, we found only one hybrid, PC(18)H2, containing an intact copy of chromosome 18 and multiple hybrids with the same breakpoint on chromosome 18q. As a result, we performed (FISH) with a chromosome 18-specific paint as well as G-banding analysis on A9.18neo cells, the original donor cell line used (data not shown). From these experiments we discovered that this cell line contained one intact copy of chromosome 18 and one truncated copy of chromosome 18, which explained why many of our hybrids had the same chromosome 18 breakpoint. To overcome this problem, we utilized the second chromosome 18 donor cell line, MCH912.1 in the construction of more PC-3 hybrids (termed HMC175 hybrids). From these experiments combined, three hybrids, PC(18)H2, HMC175-2a, and HMC175-3b containing intact copies of the exogenous chromosome 18 were selected for further characterization.

The doubling time of each microcell hybrid was compared to that of the parental cell line, PC-3 (Table 1). While some variation occurred between the doubling times of the microcell hybrids containing an intact copy of chromosome 18, in all cases it was remarkably longer than the parental CaP cell line, PC-3.

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Table 1. In vitro growth characteristics

Cell line	Doubling time (h)	Growth in soft agar <sup>a</sup>
PC-3	21	2.5 ± 1.2
PC(18)H2	37.6	0.040 ± 0.060*
HMC175-2a	41.3	0.19 ± 0.240*
HMC175-3b	66.1	0.01 ± 0.005*

The anchorage independent growth of the cell lines, as determined by growth in soft agar, was measured as well. Hybrids containing an exogenous copy of human chromosome 18 formed significantly fewer colonies in soft agar ([Table 1](#)).

The microcell hybrids were also examined for the ability to form tumors in athymic nude mice ([Fig. 1](#)). There was clonal variation between hybrids containing an intact copy of chromosome 18. However, it is apparent that in each case, while tumor growth appeared to be slower, it was not completely abolished by the introduction of human chromosome 18.

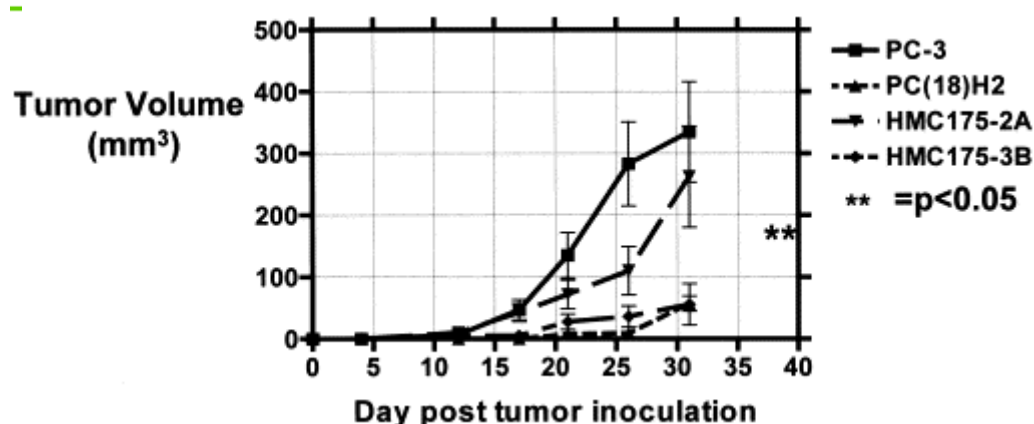


Fig. 1. Tumor growth after subcutaneous inoculation of tumor cells. Tumor volumes obtained when PC-3 parental cells or hybrid cell lines containing an intact copy of chromosome 18 were injected subcutaneously into athymic nude mice.

The parental cell line, PC-3 and the microcell hybrid cell lines, containing intact copies of the introduced chromosome 18, were also examined for the ability to form metastases in athymic nude mice after inoculation of the cells into the left cardiac ventricle. Introduction of an exogenous copy of chromosome 18 into PC-3 cells resulted in a significantly lower incidence of bone metastases as assessed by radiography, when compared to mice bearing PC-3 tumors ([Fig. 2](#)). We observed both osteolytic and osteoblastic metastases in mice inoculated with parental PC-3 cells. Representative radiographs are seen in [Fig. 3a](#). Histological slides from the same tumors seen in [Fig. 3a](#) are in [Fig. 3b](#).

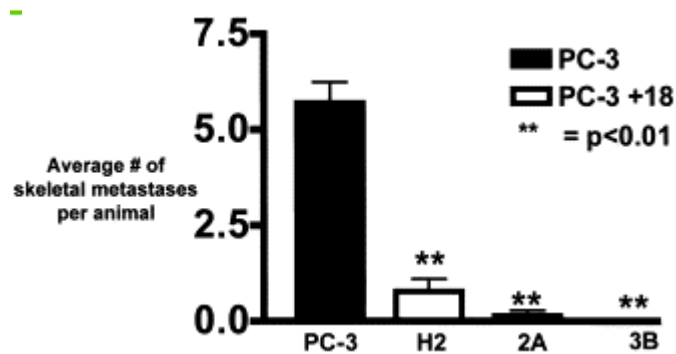
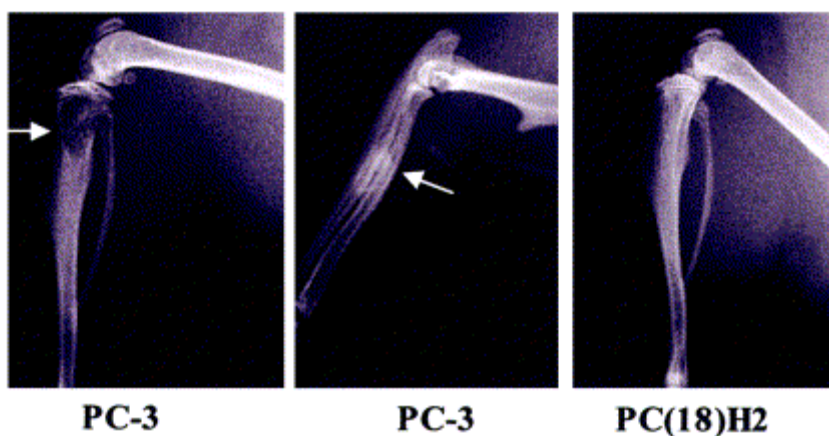


Fig. 2. Chromosome 18 reduces the number of bone metastases evident by X-ray. The average number of skeletal metastases per animal at sacrifice following intra-cardiac inoculation of PC-3 cells or hybrids containing the introduced copy of chromosome 18.

a)



b)

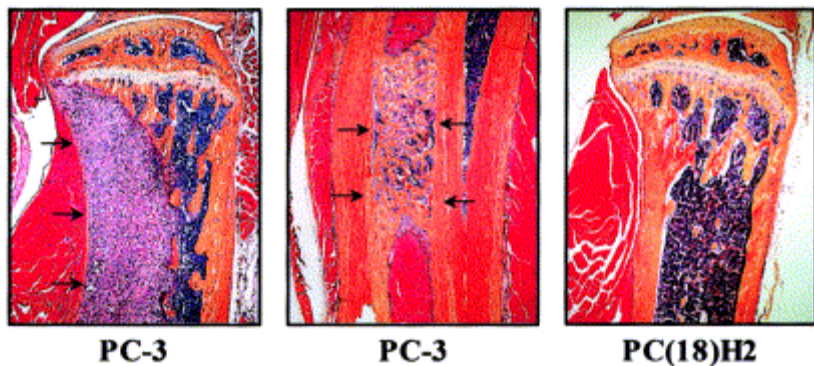


Fig. 3. (A) Radiographic appearance of representative bone metastases caused by parental PC-3 cells or PC-3 hybrids containing an intact copy of chromosome 18. Panel 1 shows a characteristic osteolytic lesion in the proximal

tibia of a mouse inoculated with PC-3 cells. Panel 2: The arrow indicates an osteoblastic lesion in the forelimb of a mouse inoculated with PC-3 cells. Panel 3: The hind limb of a mouse inoculated with a chromosome 18 containing PC-3 hybrid. There is no evidence of bone metastases in these mice. (B) Histological appearance of representative bone metastases seen in 3a. Left panel: osteolytic bone metastases characterized by osteoclastic bone destruction. Arrows indicate tumor bone interface where active bone resorption is occurring. Middle panel: osteoblastic metastases seen in 5a. arrows denote marked new bone formation adjacent to tumor cells. Right panel: tumor free bone of mouse inoculated with a chromosome 18 containing hybrid cell line, PC(18)H2.

Microcell hybrids containing an intact copy of chromosome 18 also resulted in fewer metastases to all sites in nude mice following intracardiac inoculation as assessed at sacrifice ([Fig. 4](#)). Our most significant finding is that the mice inoculated with tumor cells containing the exogenous chromosome 18 had increased survival compared to mice inoculated with parental PC-3 cells ([Fig. 5](#)). Presumably, this suppression of metastasis and concomitant increase in survival is attributable to the distal locus of loss on chromosome 18q previously identified since one copy of it appears to have been deleted in PC-3 cells [[1](#)].

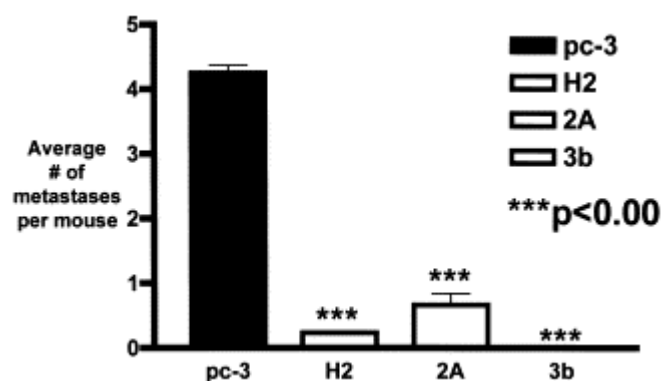


Fig. 4. Chromosome 18 reduces the number of metastases to all sites (including bone). The average number of metastases per animal at sacrifice following intra-cardiac inoculation of PC-3 cells with or without the introduced copy of chromosome 18.

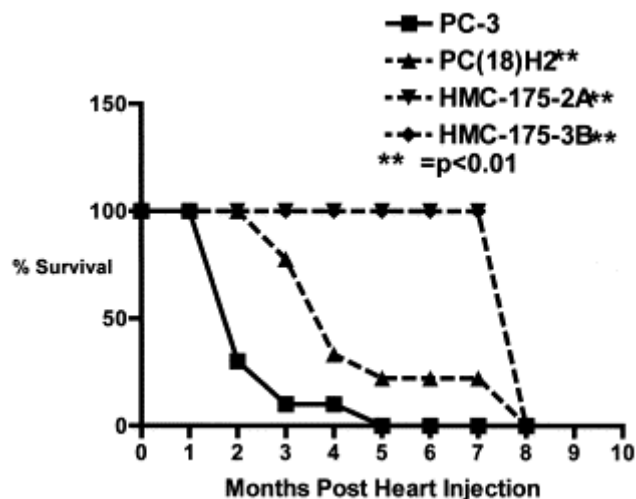


Fig. 5. Increased survival in mice bearing PC-3 microcell hybrids with an introduced copy of chromosome 18. Remaining mice inoculated with chromosome 18-containing hybrids, PC(18)H2, HMC175-2a, and HMC175-3b were sacrificed at 8 months post tumor inoculation to account for any difference in growth rates between the hybrids and the parental PC-3 cells.

## 4. Discussion

CaP is the most common tumor affecting adult males in this country (<http://www3.cancer.org/cancerinfo>). However, only a small portion of these tumors will progress to late-stage disease. To date, there is no way to distinguish between those that will metastasize and those that will remain indolent and require no immediate clinical intervention. Several investigators are defining key differences in these tumors by establishing the genetic pattern associated with the metastatic phenotype. To this end, numerous loci have been implicated as having a role in CaP progression.

Chromosome 18 has been implicated by several studies as the site of putative tumor suppressor genes involved in CaP. Others implicate chromosome 18 lesions in progression rather than initiation of CaP [1, 16, 26, 27, 28, 29, 30 and 31]. Our previous work has identified two regions of significant LOH on 18q in metastatic CaP specimens [1]. Of note, PC-3, a CaP cell line originally derived from a bone metastasis, appears to have lost one copy of chromosome 18 sequences between the genetic markers D18S1091 and D18S469 [1]. However, the gene or genes involved have yet to be identified.

The data described here provide functional evidence to support the role of chromosome 18 in the suppression of CaP progression, particularly in metastases to bone. Introduction of an intact copy of chromosome 18 resulted in a significant increase in the doubling time of PC-3 cells in vitro. In addition, similar results were seen when the cells were examined for anchorage-independent growth, a hallmark of transformed cells. The soft agar cloning efficiency of the parental PC-3 cell line was 2.5%. However, in the microcell hybrids that have retained an intact copy of the

introduced chromosome 18, the ability of the cells to form colonies in soft agar was almost completely abolished. This indicates that the long arm of chromosome 18 contains a gene or genes that are capable of suppressing the anchorage independent growth of PC-3 cells in soft agar medium, and further supports the presence of a tumor/metastasis suppressor gene on 18q.

The introduction of an intact copy of chromosome 18 appears to reduce the ability of PC-3 cells to form tumors in athymic nude mice after subcutaneous inoculation. This effect was rather mild in comparison to the effects of chromosome 18 seen in the in vitro growth assays, leading us to believe that these parameters may be under separate genetic control as has been previously proposed by Berube et al. [32] and Trent et al. [33].

The metastatic potential of PC-3 is well described. Growth in metastatic sites was initially reported in approximately 30% of nude mice inoculated subcutaneously with the cell line [18]. Furthermore, Koslowski et al. [24] and others demonstrated metastases of PC-3 after inoculation into tail vein, footpad, and spleen [20, 21, 22, 34 and 35]. Shevrin et al. [20] found bone metastases present in 20% of the mice after tail vein injection of PC-3 with occlusion of the inferior vena cava.

Parental PC-3 cells and three independent PC-3 hybrids containing an exogenous copy of chromosome 18 were inoculated directly into the left cardiac ventricle and the mice were followed by radiography at regular intervals. In this model of PC-3 metastases greater than 90% of mice inoculated with parental PC-3 cells develop metastases, to both bone and soft tissues. In contrast, less than 33% of mice inoculated with chromosome 18 containing hybrids developed metastases. These results provide strong functional evidence that a metastasis suppressor gene involved in CaP is localized to chromosome 18. In addition, mice inoculated with PC-3 hybrids containing intact copies of chromosome 18 developed significantly fewer bone metastases. Representative radiographs and histological sections of the PC-3 inoculated mice versus the healthy bones of mice receiving chromosome 18 containing hybrid cells are shown in Fig. 3.

We observed phenotypic changes in chromosome 18 containing hybrids consistent with the presence of both tumor suppressor gene and metastasis suppressor gene functions on chromosome 18. The effects on in vivo tumorigenicity are mild in comparison to the effects observed on metastasis by PC-3 cells. Because PCR analysis of PC-3 cells was limited to the long arm of chromosome 18, it is possible that these cells also harbor loss on the short arm of chromosome 18, a region not examined here. Therefore, it is also possible the effects observed on tumorigenicity are due to gene sequences on 18p. LOH on chromosome 18p has been identified in a number of tumor types including breast cancer and cervical carcinoma [36, 37, 38, 39, 40, 41 and 42]. The region on chromosome 18q, identified here as lost, overlaps with a region previously identified by LOH in metastatic CaP specimens, and thus, seems likely to harbor a metastasis suppressor gene based on the data presented here.

In addition to providing evidence for the functional role of chromosome 18 in CaP, these experiments show that PC-3 cells have the capacity to form osteoblastic metastases. Osteoblastic metastases are relevant to CaP because most skeletal metastases in CaP patients are of osteoblastic origin [43].

In summary, our results indicate that the role of chromosome 18, in particular the region of AI at 18q22.3, in CaP may be one of metastasis suppression rather than tumor suppression. We have complemented this locus in PC-3 cells. This supports the previous LOH and AI studies implicating regions of chromosome 18 in the progression of CaP. In addition, this functional evidence for the role of chromosome 18 in CaP could have wide reaching effects as chromosome 18 may also be involved in the progression of other tumor types such as gastric [44], head and neck [45], breast [46], and ovarian cancer [47]. Future studies will determine if similar results with chromosome 18 are observed with other tumor types. Finally, this work provides the first functional evidence for the role of chromosome 18 in suppression of CaP growth and metastasis. Identification of the responsible gene(s) may lead to molecular targets for drug discovery or prognostic evaluation.

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Manuscript

## **Overexpression of cadherin 7 in prostate cancer is associated with a chromosome 18q22.1 amplification**

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### **Abstract**

Prostate cancer is the second most frequent cancer reported for American men. After many decades of intense efforts, the molecular etiology of prostate cancer is still poorly understood. The recent advances in technology are proving to be quite efficient, fast and more precise in identifying alterations in chromosomal regions. Array comparative genomic hybridization is one such example of a methodology that has wide applicability in a variety of genomic disorders, including cancer. We have developed a high-resolution BAC genomic microarray for chromosome 18q21-q23 and used it to analyze DNA copy number changes in prostate cancer tissue. Using these arrays we have identified a novel amplification on chromosome 18q in approximately 55% (12/22) of primary prostate cancer. This amplicon spans a region of approximately 3.8 Mb on chromosome 18. The most prominent copy number alteration was observed at 18q22.1. Amplification of *CDH7* in prostate tumors was confirmed by FISH and real-time quantitative PCR. Finally, increased transcription of *CDH7* into mRNA in human prostate tumors was confirmed utilizing quantitative PCR.

## Introduction

The chromosomal aberrations observed in prostate cancer are numerous, and few are implicated in the progression to metastasis (reviewed by Brothman) (1). Previous studies using comparative genomic hybridization (CGH) showed that chromosome 18q is frequently lost in prostate cancer (1). It is understood that at least two genes on chromosome 18q, *DCC* and *DPC4*, show allelic imbalance (AI) in a variety of epithelial cancers including prostate cancer (2). However, highest incidence of AI on chromosome 18q is observed in metastatic prostate cancer (3). Considering the low frequency of mutations observed for these two genes in primary prostate cancer, their functional significance in prostate tumorigenesis is unknown. The amplification of chromosome 18q, albeit to a lower frequency compared to its loss, has also been observed in prostate cancer (4) and other cancer types (5,6). *BCL2*, an anti-apoptotic gene located on 18q, does not necessarily show simultaneous increase in the copy number in these cancers, indicating that there are other targets of amplification at 18q.

The application of array CGH to study genomic changes in cancer has confirmed previously known chromosomal changes as well as identified novel candidate genes (7). Both bacterial artificial chromosome (BAC) microarray (8) and cDNA microarray (9) have been used to detect DNA copy number changes in genome-wide analyses on prostate cancer, resulting in the identification of several novel, as well as previously known genetic alterations. However, the average resolution of whole genome BAC microarrays used in earlier studies to identify genetic changes in prostate cancer was approximately 1 Mb (8). These arrays are less capable of detecting single BAC clone changes from a specific chromosomal segment, as compared to arrays constructed with genomic clones from the tiling path. To further characterize the region on chromosome 18q for copy number variations, we have developed a high-resolution tiling path resolution BAC microarray spanning 18q21 to 18q23. This microarray identified a novel chromosomal aberration on 18q in primary prostate cancer.

## Materials and Methods

### Tumor samples

All studies involving human samples were approved by the Institutional Review Board at the University of Texas Health Science Center at San Antonio. The prostate tumor samples were from patients who had undergone prostatectomies at the University Hospital, San Antonio, TX.

### Cell lines

The cell line PZ-HPV-7 was purchased from American Type Culture Collection (ATCC) and grown following the recommended conditions for culturing.

### DNA extraction

**Paraffin Embedded Tissue:** DNA was extracted from four 10  $\mu$ m tissue sections of paraffin embedded specimens. The tumor tissue was microdissected from these sections using a corresponding H&E stained slide as the template. After dewaxing

using xylene, tumor tissue was digested in lysis buffer (10 mM Tris-HCl, pH 8, 0.5% SDS and 20 µg/ml RNase) containing 20 µg/ml Proteinase K (Roche Diagnostics, Indianapolis, IN). Once the digestion was completed, DNA was extracted with phenol/chloroform. Twenty µg of glycogen (Roche Diagnostics, Indianapolis, IN) was added to enhance the precipitation of DNA. The DNA pellet was washed once in 75% ethanol, resuspended in 50-100 µl of sterile water, quantified with a fluorometer and quality tested by electrophoresis through a 1% agarose gel.

### **Array CGH**

From the chromosome 18 tiling path (UCSC genome browser, 2001-2003 freezes), 165 BAC clones were selected and PCR-verified. The BAC DNA was extracted using the Plasmid Maxi kit (Qiagen, Valencia, CA) and spotted onto slides in duplicate by Spectral Genomics (Houston, TX). The slide format was based on the company's Constitutional Chip<sup>TM</sup> array. One µg each of tumor DNA and normal male DNA (Promega, San Luis Obispo, CA) was sonicated (Sonic Dismembrator, Fischer Scientific, Pittsburgh, PA) and labeled with Cy5 and Cy3 (Perkin-Elmer, Boston, MA) using the Bioprime DNA labeling system (Invitrogen, Carlsbad, CA). The labeling, hybridization and washing of the slides were done as recommended by Spectral Genomics, except that the washes were carried out at 42°C. The data processing of Cy5/Cy3 ratios was accomplished using SpectralWare, version 2.1 (Spectral Genomics).

### **Fluorescence *In Situ* Hybridization (FISH)**

For normal metaphase FISH, a primary peripheral blood culture from a normal male donor was used. For paraffin embedded tissue (5 µm thickness), the paraffin pretreatment reagent kit II (Vysis Inc., Downers Grove, IL) was used, according to the manufacturer's protocol. Amplification of the signal was performed on both the normal metaphase and paraffin embedded tissue. A previously described protocol for FISH was used for performing the hybridization (10). The DNA from BAC clones RP11-775G23 and RP11-389J22 was used as probes. A centromeric probe specific for human chromosome 18 (Vysis Inc., Downers Grove, IL) was used as the control. Four,6-diamidino-2-phenylindole (DAPI) was used as the counter stain. Slides were viewed using a Zeiss Axioplan 2 fluorescence microscope and the images captured using Genus software (Applied Imaging, San Jose, CA).

### **Real-time quantitative PCR**

The TaqMan primer-probe sets were designed using Primer Express 2 software (Applied Biosystems, Foster City, CA).

*Analysis on genomic DNA:* For testing DNA copy number of *CDH7*, the primer sequences used were,

CDH7A: 5'CAAAGACAACACAGCCTCAATACTG3'-Forward

CDH7B: 5'TGGCAGATAGTAAACTGATTGTTCT3'-Reverse

The primer sequences for the internal control (11) were,

Albumin: 5'AGGGTAAAGAGTCGTCGATATGCT3'-Forward

Albumin: 5'CAATCTCAACCCACTGTCAGCTA-Reverse

The TaqMan probes used were,

6FAMTCCGGAAGCCGTTTCTCCTGGTAMRA and

6FAMCAAACGCATCCATTCTACCAACTTGAGCATTAMRA for *CDH7* and albumin, respectively.

*Expression Analysis:* For real-time RT PCR, the primer sequences used for *CDH7* were,

*CDH7B*: 5'TGGCAGATAGTAAACTGATTGTTTCCT3'-Reverse

*CDH7C*: 5'CAACAAATAACCACAACCTTTTCATTG3'-Forward

The same *CDH7* probe was used to test both the DNA copy number and the expression levels of *CDH7* mRNA. For the internal control, the TATA-box binding protein expression assay (Applied Biosystems, Foster City, CA) was used in gene expression analysis.

All reactions were conducted in 384-well plates using the 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) following the protocol recommended by the manufacturer. TRIzol reagent (Invitrogen, Carlsbad, CA) was used to extract total RNA from frozen sections of the tumor. First strand cDNA was synthesized from these RNAs using the TaqMan Reverse Transcription kit (Applied Biosystems, Foster City, CA) and were used as templates in reactions consisting of 1X TaqMan Universal PCR Master mix (Applied Biosystems, Foster City, CA), 900 nM of *CDH7B/CDH7C* primers and 200 nM *CDH7* probe. For the endogenous control TATA-box binding protein (TBP) expression assay (Applied Biosystems, Foster City, CA) was used. To test DNA copy number, 20 ng of genomic DNA, 900 nM of *CDH7A/CDH7B* primers and 200 nM probe were used for *CDH7*; the control albumin had similar conditions except that the albumin primers were used at a concentration of 300 nM each. The PCR cycling conditions consisted of 50° C for 2 min; 95° C for 10 min; and 40 cycles each of 95° C for 15 sec and 60° C for 1 min. The results were analyzed using the SDS software (Applied Biosystems, Foster City, CA).

## Results

### Frequent amplification at 18q22.1 detected by array CGH

We constructed a tiling path resolution BAC microarray for the chromosomal region 18q21-q23. This region was represented by 165 PCR-verified BAC clones. We analyzed 22 primary prostate tumors by array CGH. The most prominent copy number alteration occurred at 18q22.1, which showed amplification in 55% of prostate tumors (12/22) (Fig. 1). This amplicon spans the region of chromosome 18 contained in BACS RP11-775G23 through RP11-543H23 (approximately 3.8 Mb). There are two known genes, cadherin 7 (*CDH7*) and cadherin 19 (*CDH19*) present in this amplified region. The highest level of amplification observed on chromosome 18 corresponded to BAC clone, RP11-775G23 (Fig. 1). Interestingly, the 5' end of *CDH7* is coded on the DNA contained in RP11-775G23. The normal male/normal female hybridization exhibited diploid copy number across this region, implying that the amplification is tumor specific.

### Verification of amplification at 18q22.1

Since the most striking copy number variation was observed with the BAC RP11-775G23, we further verified the amplification detected by array CGH using FISH and real-time quantitative PCR. We tested two prostate tumors that exhibited high-level amplification using FISH, specimens N40 and N41, and both tumors showed more than the normal two copies of RP11-775G23 and RP11-389J22 (Fig. 2). Furthermore, the amplification was tumor-specific because the adjacent normal cells contained only two copies of the probe, RP11-775G23. To quantitate the fold differences in the copy number of *CDH7* between the tumor and normal cells, real-time quantitative PCR was performed on the genomic DNA from the tumors that were used for array CGH analyses. The copy number of the *CDH7* gene in the prostate tumors ranged between two and seven. For the majority of samples, the gene copy number of *CDH7*, as detected by quantitative PCR, correlated with the degree of amplification of RP11-775G23 detected by array CGH in the corresponding tumor section. These findings are consistent with the hypothesis that *CDH7* is a key target for amplification in prostate cancer. We, however, did not find any correlation between *CDH7* copy number and Gleason score (Table 1).

### Quantification of *CDH7* mRNA

We tested RNA extracted from microdissected prostate tumors to verify whether the genomic amplification of the gene has any impact at the transcriptional level of *CDH7*. As expected, we detected three- to eight-fold overexpression of *CDH7* in prostate tumors compared to the normal adjacent tissue (Fig. 3). The cell line PZ-HPV-7, derived from normal prostate epithelial cells, showed very low *CDH7* expression. The expression of *CDH7* was several hundred-fold higher in the tumors, compared to PZ-HPV-7 (Fig. 3). Together, array CGH, FISH and real-time quantitative PCR show amplification of *CDH7* gene in prostate cancer and this amplification correlates with increased expression of *CDH7*.

### Discussion

The cadherins are a large family of transmembrane glycoproteins belonging to the immunoglobulin superfamily that mediate cell-cell adhesion and communication. Since the cadherins are involved in forming cell-cell adhesion, altered expression could lead to increased cell motility, and in cancer this results in metastasis. The changes in the expression patterns of cadherins can contribute to the tumor progression by altering the normal cell-matrix interactions (12). In prostate cancer, a transient downregulation of E-cadherin expression was noted in localized cancer, while the metastatic cancers showed normal expression for E-cadherin (13). A 'switch' in the expression pattern from a normal epithelial E-cadherin type to the mesenchymal N-cadherin has been shown in breast cancer (14), prostate cancer (15) and melanomas (16).

In prostate cancer cell lines, a complex pattern of cadherin expression has been observed; the levels of each member, including E-cadherin, N-cadherin, P-cadherin, R-cadherin, K-cadherin and cadherin-11, have differing ratios in a particular cell

line (17). Previous studies, however, have not investigated the expression of *CDH7*, *CDH19* and *CDH20*, located on chromosome 18q22-q23, (18,19). Even though the three genes, *CDH7*, *CDH19* and *CDH20*, are clustered in a region of about 5.7 Mb on chromosome 18q, the expression of each gene is quite varied in different tissues. Our study reports frequent overexpression of *CDH7* in primary prostate cancer, compared to the normal prostate tissue.

The influence of inappropriate expression of nonepithelial cadherins is exhibited by breast cancer cells, where overexpression of N-cadherin resulted in increased invasiveness and metastasis (14). In normal cells, the expression of *CDH7* is restricted to the brain, prostate and testis (18). From our analysis using quantitative RT PCR, it appears that *CDH7* expression is increased to eight-fold in prostate tumors relative to normal prostate. The type II cadherin family, to which *CDH7* belongs, differs from type I cadherins, including E-cadherin, N-cadherin and P-cadherin, by the absence of a histidine-alanine-valine (HAV) motif within the first cadherin repeat (19). It has been shown that the lack of the HAV domain in type II cadherins does not compromise their ability to form cell-cell adhesion complexes (19). The type I cadherins mainly show homotypic interaction when forming cell-cell adhesion (20). Unlike type I cadherins, type II cadherins, including *CDH7* and *CDH14*, have been shown to interact heterotypically in a cell aggregation assay using mouse fibroblasts that lack E-cadherin, which were transfected with various human type II cadherins (21). Currently, it is not known whether such heterotypic interactions occur *in vivo*, and what might be the consequences of these interactions. In our array CGH analyses, we did not find any copy number change for *CDH20*, which is located approximately 5 Mb proximal to *CDH7*. *CDH19*, which has a more ubiquitous pattern of expression (18), showed only low-level amplification by array CGH.

Previously, we reported the presence of AI at two distinct regions on chromosome 18q in metastatic prostate cancer (22). Whether the amplification of one allele is the causative factor, is yet to be tested. The loss of E-cadherin expression and concomitant overexpression of mesenchymal cadherins, N-cadherin and *CDH11* have been reported in high grade prostate tumors (15). The mechanism by which N-cadherin and *CDH11* are upregulated in the tumors has not been studied. Interestingly, N-cadherin is located at chromosome 18q11. About 30% of 37 hormone-refractory prostate tumors exhibit gain of 18q12 by CGH (4). A mutually exclusive pattern of expression has been seen for *CDH7* and E-cadherin in human melanoma cell lines derived from tumor biopsies from patients with primary or metastatic melanoma (23). This study reported that normal melanocytes did not express *CDH7*, while melanoma cell lines expressing *CDH7* grew tumors in nude mice, thus indicating that *CDH7* plays a crucial role in melanoma tumorigenesis. Further exploration is needed to determine whether the upregulation of *CDH7* in prostate cancer interferes with normal cell-adhesion and signaling.

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Table 1. Prostate tumor samples with various pathological grades showing amplification at chromosome 18q22.1

Tumor	Age	Gleason's	Gleason	Stage	Amplification at 18q22.1
N10	66	6	6	T2CNXMX	Medium
N12	64	6	6	T2CNXMX	Medium
N15	72	7	7	T2CNXMX	Medium
N22	59	7	7	T2CN0MX	High
N26	56	6	6	T2CNXMX	Low
N30	73	5	5	T2CNXMX	Medium
N31	72	6	6	T2CNXMX	Medium
N32	54	8	8	T2CN0MX	Low
N34	52	7	7	T3BN0MX	Normal
N35	56	7	7	T2CNXMX	Low
N36	62	6	6	T2CNXMX	Medium
N37	62	9	9	T3AN0MX	High
N38	59	6	6	T2CNXMX	Medium
N39	60	7	7	T3NXMX	Low
N40	70	7	7	T2CNXMX	High
N41	67	7	7	T3BN0MX	High
N42	67	8	8	T2CN0MX	Low
N43	65	5	5	T2BNXMX	Low
N44	53	7	7	T2BNXMX	Medium
N45	55	9	9	T2BN0MX	Low
N47	71	9	9	T3BN0MX	Low
N49	56	9	9	T2CNXMX	Low

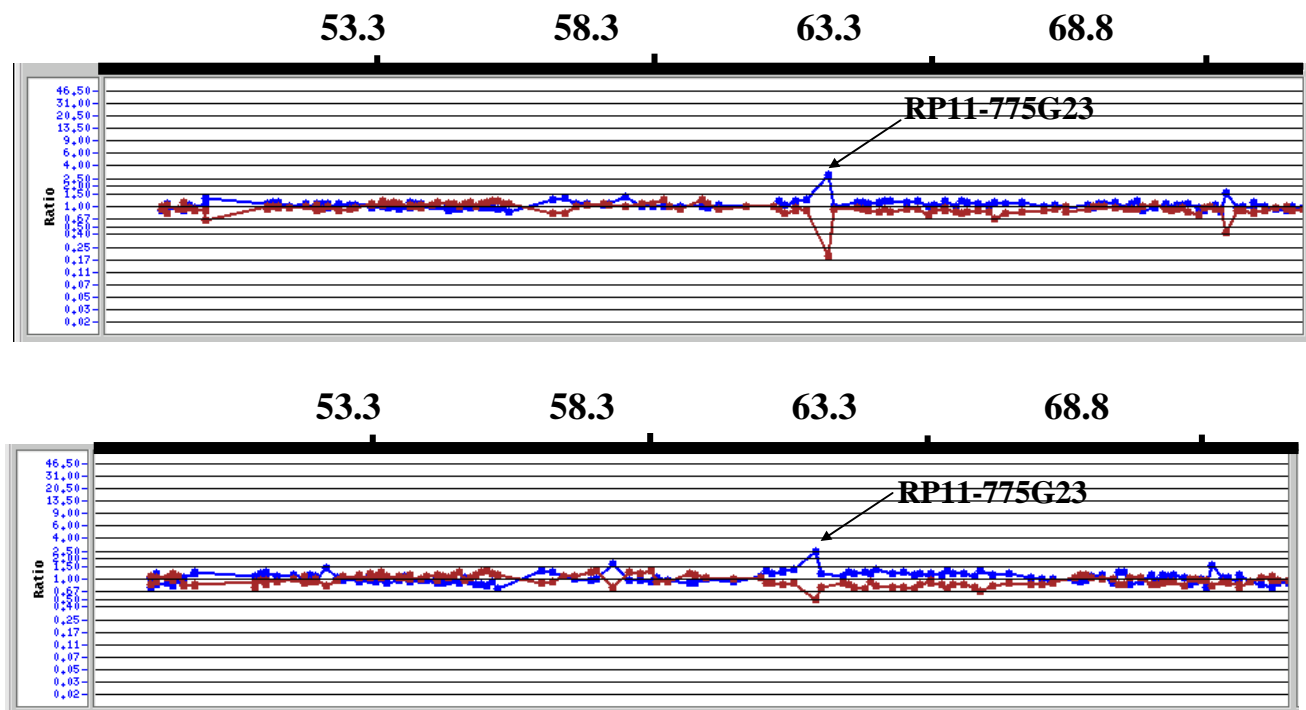
## Figure Legends

Fig. 1. Array CGH on prostate tumor samples showing amplification of the genomic region contained within RP11-775G23 at chromosome 18q22.1. Genomic DNA from prostate tumors was labeled with Cy5 in experiment #1 (represented by blue curve) and normal male reference was labeled with Cy3. Dye-reversal was done for the same pair of DNAs in experiment #2 (represented by red curve). The X-axis represents the cytogenetic position of each BAC clone (in Mb) and Y-axis represents  $\log_2$  ratio of Cy5/Cy3.

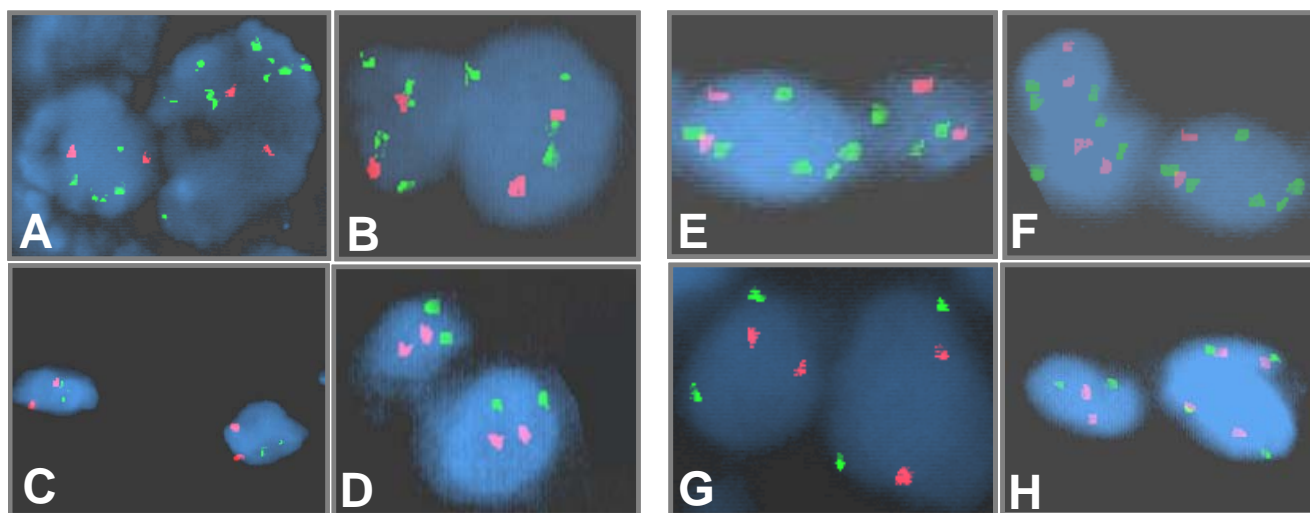
Fig. 2. Validation of the amplification at chromosome 18q22.1 using FISH on paraffin-embedded tumor sections. A probe derived from BAC, RP11-775G23 (FITC) detects multiple copies of this region in the prostate tumors (A&B), while corresponding adjacent normal cells (C&D) show only two copies. The control probe (spectrum orange) was a human chromosome 18-specific centromeric probe. A probe derived from RP11-389J22, the clone flanking RP11-775G23, also shows amplification in the tumors (E&F), while adjacent normal cells (G&H) show only two copies.

Fig. 3. Real-time quantitative PCR analysis of *CDH7* in prostate tumor samples. A. Relative expression levels of *CDH7* in four different tumors as compared to the matched adjacent normal tissue. B. *CDH7* expression in six different tumors relative to the prostate epithelial cell line, PZ-HPV-7. The expression level of *CDH7* in PZ-HPV-7 is considered as 1. The error bars depict the values for standard deviation.

Figure 1

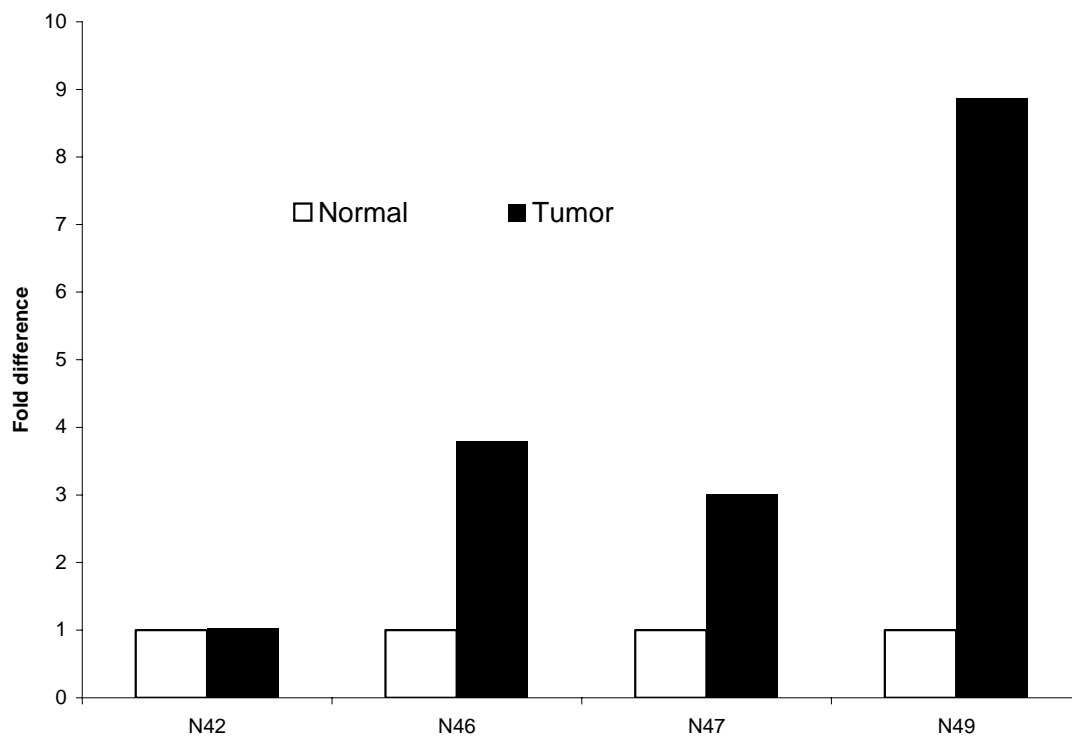


**Figure 2**



**Figure 3**

**A**



**B**

